EXHIBIT 1

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PURIFICATION OF AN ACIDIC α-D-MANNOSIDASE FROM *ASPERGILLUS SAITOI* AND SPECIFIC CLEAVAGE OF 1,2-α-D-MANNOSIDIC LINKAGE IN YEAST MANNAN

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Summary

An acidic α-D-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) has been isolated from culture filtrate of *Aspergillus saitoi*. The extracellular α-mannosidase was homogeneous in polyacrylamide gel electrophoresis. The molecular weight of the enzyme was 51 000 and the isoelectric point pH 4.5. The purified enzyme has a pH optimum of 5.0, a K_m of 0.45 mM with baker's yeast mannan and has no activity towards *p*-nitrophenyl-α-D-mannoside. The mode of action of the enzyme has been studied with baker's yeast mannan and saké yeast mannan. The enzyme cleaves specifically the 1,2-α-linked side chain, producing free mannose.

Introduction

The importance of α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) in the degradatory system of higher eukaryotic cells is well known, since a deficiency in the enzyme results in the lethal disease, mannosidosis, which occurs in both humans [1] and Angus cattle [2—4].

Jones and Ballou [5—7] reported that the extracellular exo-1,2-1,3-α-D-mannosidase (1,2-1,3-α-D-mannan mannohydrolase, EC 3.2.1.77) from *Arthrobacter* GJM-1 cleaves the 1,2-α- and 1,3-α-linked side chains from baker's yeast (*Saccharomyces cerevisiae*) mannan at pH 6.8, producing free mannose leaving a resistant polymer containing mainly 1,6-α-linkages. On the other hand,

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although extracellular 1,2-α-D-mannosidase has been characterized from *Aspergillus niger*, the 1,2-α-mannosidase does not hydrolyze baker's yeast mannan and *p*-nitrophenyl-α-mannoside [8]. The 1,2-α-D-mannosidase from *A. niger* is highly specific for synthetic 2-*O*-α-D-mannosidic linkages of 2-*O*-α-D-mannobiose and 2-*O*-α-D-mannotriose at pH 4.8. Another α-mannosidase from *A. niger* appeared to be specific for 4-*O*-α- and 6-*O*-α-D-mannosidic linkages to the adjoining mannose or *N*-acetyl-glucosamine residue [9]. The latter α-mannosidase from *A. niger* is highly specific for *p*-nitrophenyl-α-D-mannopyranoside and 4-*O*-α-mannopyranosyl-α-D-mannopyranoside [9].

The present report deals with isolation of an 1,2-α-D-mannosidase from *A. saitoi* and mode of action on baker's yeast mannan and saké yeast mannan, producing free mannose at pH 5.0. To the best of our knowledge no mannosidase with similar specificity has been reported earlier.

Materials and Methods

Enzyme substrates

Baker's yeast mannan was purchased from Sigma (Lot. 77C-0238). Saké yeast mannan [10] was a generous gift from Dr. T. Kumagai, Research Inst. of Brewing, Tokyo. *p*-Nitrophenyl-α-D-mannoside (CH-8157) was from Wako Pure Chemicals, Osaka.

To obtain a crude enzyme product

A. saitoi MR R 3813 (now designated as ATCC 14332) was obtained from Prof. H. Iizuka, Tokyo Science University. The mold *A. saitoi* was maintained on Koji agar slopes at 10°C and transferred twice per year. Czapeck's medium and the modified Czapeck medium, containing glucose and peptone, can be substituted for the Koji agar slope.

For the enzyme production, cultivation of *A. saitoi* was carried out in solid culture (Koji-culture). Wheat bran (30 g) was thoroughly mixed with 21 ml distilled water in a 1000-ml Erlenmeyer flask and autoclaved (1 kg/cm^2, 30 min). The sterilized bran was inoculated with spores of *A. saitoi*, and then kept at 30°C for at least 96 h. The mold grows satisfactorily on this medium. The production of carboxyl (acid) proteinase having optimal pH 2.5—3.0 (11) falls by 70 to 50% if the cultural growth is more than 100 h. The flasks were shaken twice or three times a day to redistribute the medium and produce better temperature control.

The major part of the α-mannosidase produced in Koji-culture in the flask was extracted with 200 ml 0.1 M sodium acetate/HCl buffer (pH 3.0). The mixture was adjusted to a pH 5 with 1 HCl and allowed to stand in the cold for 5 h or more.

After extraction from cultures, the culture filtrates were pooled and the pH was adjusted to 5. The 60—80% $(NH_4)_2SO_4$ precipitate was collected at 4°C. This α-mannosidase preparation was free of carboxyl proteinase activity.

The enzyme precipitate was dissolved with 10 vol. 0.01 M sodium acetate acetic acid buffer (pH 5.0). The insoluble materials were removed by centrifugation (6000 × *g*, 10 min). The α-mannosidase is concentrated by adding cold (4°C) ethyl alcohol to a final concentration of 65%. The crude alcohol precipi-

tate was fractionated using centrifugation at 12 600 × *g* for 15 min.

For the preparation of crude enzyme solution, the crude alcohol precipitate was dissolved in 0.01 M sodium acetate/acetic acid buffer (pH 5.0)/0.2 M NaCl. The isoluble materials were removed by centrifugation at 12 600 × *g* for 15 min. The crude enzyme solution was applied to a column of Sephadex G-100 (see figure legends).

Enzyme assays and protein determination

p-Nitrophenyl-α-D-mannosidase activity was determined according to the published method [9] at pH 5.0. 1 kat of the activity was defined as the enzyme required to liberate 1 mol of *p*-nitrophenol/s at 30°C and pH 5.0.

For an α-mannosidase determination 100 μl 2% baker's yeast mannan dissolved in 0.1 M sodium acetate/acetic acid buffer, pH 5.0, were added to 50 μl of the enzyme solution. After 24 h at 30°C the reaction was stopped by heating to 100°C for 2 min. 15 μl of the reaction mixture were applied as a spot to Toyo No. 51 filter paper. For descending paper chromatography, the following solvent was used: *n*-butanol/pyridine/water (6 : 4 : 3 v/v). Sugar spots were detected with the silver sodium hydroxide dip reagent [12]. For the crude enzyme preparation, the stained spot of liberated mannose on the paper chromatogram was determined with Densitron Model-Pan densitometer (Joko-Sangyo Co.).

1,2-α-D-Mannosidase activity of the preparation was also determined with the mannose released from baker's yeast mannan and saké yeast mannan by the method of Somogyi-Nelson [13]. Free mannose on the paper chromatogram was cut out and eluted with distilled water. Mannose in the eluate was evaporated in vacuo and the residue was dissolved in 1 ml distilled water. 250 μl of this solution were added to 250 μl of the freshly prepared alkaline copper reagent, and the mixture was heated at 100°C for 10 min and immediately cooled in an ice-water bath for 3—10 min. After addition of 250 μl Nelson reagent [13] and 2.5 ml distilled water, the mixture was stored for 15 min. The absorbance was measured at 660 nm in a Hitachi model 101-10 spectrophotometer or Shimazu model ultraviolet 100-02 spectrophotometer, the amount of mannose liberated was determined from the standard D-(+)-mannose solutions.

1 kat 1,2-α-D-mannosidase was defined as the amount of enzyme required to liberate 1 mol mannose from baker's yeast mannan or sake' yeast mannan per s at 30°C and pH 5.0.

Protein was determined by the method of Lowry et al. [14], using bovine serum albumin (fraction V, Daiichi Pure Chemicals Co.) as a standard.

Column chromatography

All experiments were carried out at 4°C. Details are described in the legends to the figures.

Enzyme characterization

Polyacrylamide gel electrophoresis was performed at 30°C with the standard pore formulation at pH 9.4 and 3 mA constant for gel [15].

Molecular weight determination was performed using gel filtration on Sephadex G-100. The column (2 × 67 cm) was eluted with 0.01 M sodium

acetate/acetic acid buffer (pH 5.0)/0.2 M NaCl. The following preparations were used at a concentration of either 5 mg/ml or 20 mg/ml: from Miles Laboratories, myoglobin (M_r 17 000); General Biochemicals, porcine pepsin (M_r 34 600); Miles Laboratories, egg albumin (M_r 45 000) and Miles Laboratories, human hemoglobin (M_r 64 000).

For isoelectric focusing [16], an LKB electrofocusing column (110 ml vol.) was used. Ampholines, pH 3.5—5.0 (LKB Produkter AB, Fack, Sweden, Lot 1809—111), were incorporated into a sucrose gradient to 1%. The focusing time was 60 h with the column at 4°C and 500 V. Fractions of 1.6 ml were collected. Absorbance at 280 nm in each fraction was estimated using microcuvettes. Immediately after the pH had been determined using a Radiometer titrater TTT-type pH meter, the purified enzyme was dialysed against 0.01 M acetate buffer (pH 5.0) to remove the ampholines and then stored at 4°C.

Gas-liquid chromatographic analysis of enzymic digests

Yeast mannan (5 mg) was dissolved in 0.5 ml 0.01 M sodium acetate/acetic acid buffer (pH 5.0). The reaction was started by adding 100 µl 1,2-α-mannosidase at 30°C. After, the reaction was stopped by heat treatment at 100°C for 2 min, the reaction mixture was dialyzed against tap water for 24 h and then lyophilized.

The digested mannans were dissolved in 0.5 ml dimethyl sulfoxide and methylated by the method of Hakomori [17]. The methylated saccharides were methanolyzed by treatment with 5% methanolic hydrogen chloride at 80°C for 16 h. The methanolyzate was neutralized with silver carbonate, filtered and concentrated. The syrupy residues were dissolved in 0.2 ml methanol and analyzed by gas-liquid chromatography. Methanol solution (25 µl) was injected into a column (3 × 2000 mm) of 2% neopentyl glycolsuccinate on hexamethyldisilazane treated Chromosorb W (80—100 mesh), followed by isothermal separation at 160°C. Carrier gas (N_2) flow rate was 30 ml/min. Peaks were identified by comparison with the experimental data of retention time described by Bhattacharjee and Gorin [18].

Results

Purification of 1,2-α-D-mannosidase

The crude enzyme solution obtained from the crude alcohol precipitate was chromatographed on a column of Sephadex G-100 (Fig. 1). A descending paper chromatogram of the product of the eluted α-mannosidase on baker's yeast mannan is shown in Fig. 2. Active fractions from No. 50 to 56 were collected. The specific activity was 1.1 mkat/kg enzyme, at this stage. The 1,2-α-D-mannosidase could be separated from *p*-nitrophenyl-α-D-mannoside. Most *p*-nitrophenyl-α-D-mannosidase activity emerged faster than that of 1,2-α-D-mannosidase (Fig. 1). The *p*-nitrophenyl-α-D-mannosidase studied in this paper behaved exactly as the α-mannosidase from *A. niger* reported by Matta and Bahl [9]. The carboxyl (acid) proteinase activity [11] emerged slower than that of 1,2-α-D-mannosidase, because the molecular weight of the carboxyl proteinase was 35 000.

The enzyme eluted from Sephadex G-100 was applied to a column of DEAE-

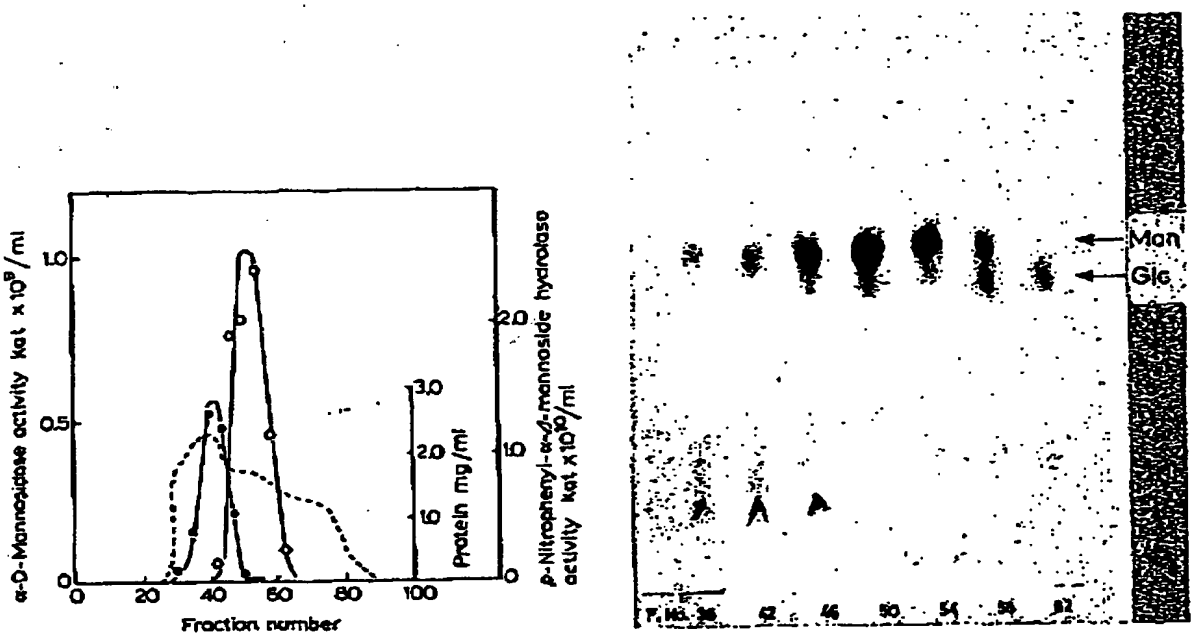


Fig. 1. Gel filtration of crude enzyme preparation from the alcohol precipitate of *A. saitoi* on Sephadex G-100. Column: 5 X 60 cm. Eluent: 0.1 M sodium acetate acetic/acid buffer (pH 5.0)/0.2 M NaCl. Flow rate: 20 ml/h. Fraction volume: 10 ml. - - - - - -, protein; o———o, α-mannosidase activity and •———•, *p*-nitrophenyl-α-D-mannosidase activity. The dark bar fractions were collected.

Fig. 2. Descending paper chromatogram of the enzymic product of the α-mannosidase from *A. saitoi* on baker's yeast mannan. The numbers in the paper chromatogram indicate the enzyme fractions separated from Sephadex G-100 gel filtration procedure, as shown in Fig. 1. Man, mannose. Glc, glucose.

Sephadex A-50 (Fig. 3). The specific activity was 7.7 mkat/kg enzyme, at this stage. The eluted enzyme was applied to a column of SP-Sephadex C-50 (Fig. 4). The specific activity of the purified 1,2-α-D-mannosidase was 11.5 mkat/kg enzyme.

The results of the purification are summarized in Table I.

Enzyme characterization

The optimum pH with baker's yeast mannan was 5.0 as described in Fig. 5.

The purity of the final step product from the SP-Sephadex C-50 was checked by polyacrylamide gel electrophoresis. The protein migrates as a single band on gel electrophoresis at pH 9.4. An isoelectric point of the 1,2-α-D-mannosidase was 4.5 on isoelectric focusing. According to the gel filtration molecular weight value of the 1,2-α-D-mannosidase was determined to be 51 000. The purified preparation was free from the carboxyl proteinase activity having an isoelectric point pH 4.08.

About 70% or more of the 1,2-α-D-mannosidase activity was stable at a temperature of 4°C for 40 days in 0.01 M acetate buffer (pH 5.0)/0.2 M NaCl.

Solution of freeze-dried 1,2-α-D-mannosidase in distilled water or in 0.01 M sodium acetate/acetic acid buffer (pH 5.0) showed 90% or more of the activity.

The 1,2-α-mannosidase activity in 0.01 M sodium acetate acetic acid buffer (pH 5.0) was stable at 45° C for 10 min. The activity decreased by about

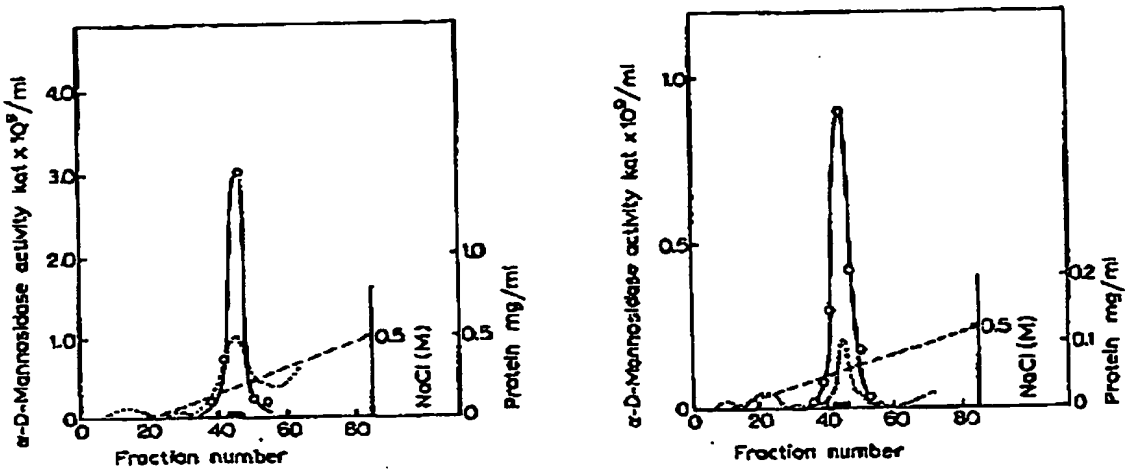


Fig. 3. DEAE-Sephadex A-50 chromatography of *A. saitoi* α-mannosidase from Sephadex G-100 run. The column (1 X 50 cm) was equilibrated with 0.01 M sodium acetate/acetic acid buffer (pH 5.0). Elution was performed with 0.01 M acetate buffer (pH 6.0) with increasing concentration of salt gradient. Flow rate: 10 ml/h. Fraction volume: 5 ml. ·····, protein; o———o, α-mannosidase activity and - - - - - -, NaCl. The dark bar fractions were collected.

Fig. 4. SP-Sephadex C-50 chromatography of *A. saitoi* α-mannosidase from DEAE-Sephadex from DEAE-Sephadex A-50 run. The column (1 X 50 cm) was equilibrated with 0.01 M sodium acetate/acetic acid buffer (pH 4.0). Elution was performed with 0.01 M sodium acetate/acetic acid buffer (pH 4.0) with increasing concentration of salt gradient. Flow rate: 10 ml/h. Fraction volume: 5 ml. ·····, protein; o———o, α-mannosidase activity and - - - - - -, NaCl. The dark bar fractions were collected.

50% at 60°C after 10 min. 90% or more of the activity was lost at 70°C for 10 min, probably due to thermal denaturation of the enzyme.

Specificity of an α-D-mannosidase from A. saitoi

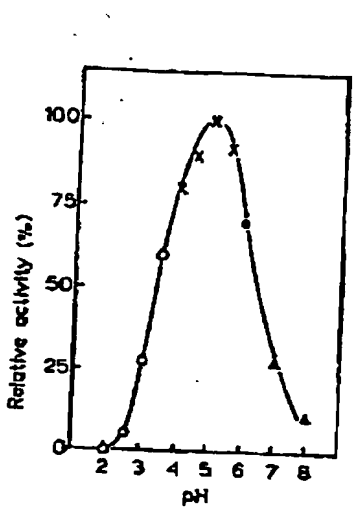
As shown in Fig. 2, an α-D-mannosidase from *A. saitoi* hydrolyzed baker's yeast mannan, producing free mannose. The enzymic digest, mannose, obtained from saké yeast mannan was identical with that of the baker's yeast mannan. No oligosaccharides obtained by enzymic digestion were shown on the paper chromatogram (Fig. 2).

The reaction mixture was dialyzed, lyophilized and then analyzed by gas-liquid chromatography. Fig. 6 shows a chromatogram of the methanolyzate of the premethylated baker's yeast mannan. The results in Table II show that the rapid decrease of methyl-3,4,6-tri-*O*-methyl-D-mannoside was found in the

TABLE I

PURIFICATION OF *ASPERGILLUS SAITOI* 1,2-α-D-MANNOSIDASE

Fraction	Vol. (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (mkat/kg)	Purification	Yield (%)
Crude alcohol precipitate	—	—	—	0.2	1	100
Sephadex G-100	65	90	85	1.1	6	35
DEAE-Sephadex A-50	32	32	4.1	7.7	39	12
SP-Sephadex C-50	25	18	1.6	11.5	58	7



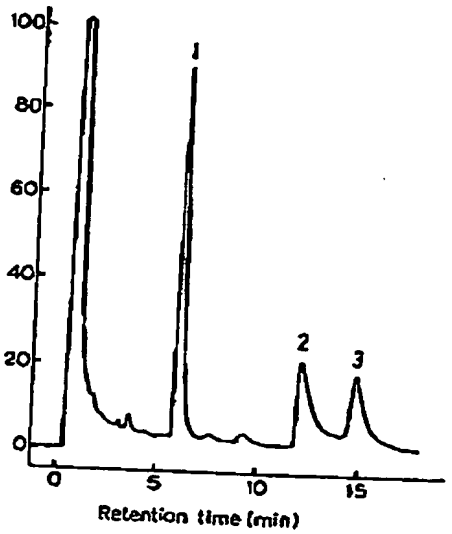


Fig. 5. Effect of pH on the activity of α-D-mannosidase from *A. saitoi*. ○, sodium acetate/HCl buffer. ×, Sodium acetate/acetic acid buffer. ●, Sodium phosphate dibasic hydrate/citric acid buffer. ▲, Potassium phosphate monobasic/NaOH buffer.

Fig. 6. Gas chromatogram of methanolyzate of premethylated baker's yeast mannan. 1, methyl-2,3,4,6-tetra-*O*-methyl-α-D-mannoside. 2, methyl-3,4,6,-tri-*O*-methyl-α-D-mannoside. 3, methyl-2,4,6,tri-*O*-methyl-α-D-mannoside.

enzymic digest with the α-D-mannosidase from *A. saitoi*. The data show that an α-D-mannosidase from *A. saitoi* rapidly hydrolyzed the single mannose unit from the nonreducing termini of the 1,2-α-D-linked side chain from baker's yeast [5] and saké yeast [10] mannan at pH 5.0 (Table II), producing free mannose (Fig. 2). Baker's yeast mannan is a better substrate for *A. saitoi* α-D-mannosidase than saké yeast mannan. The published data indicated that the baker's yeast mannan was a highly branched polysaccharide with 1,2-α- and 1,3-α-linked side chain attached to an 1,6-α-linked backbone [6,19,20]. It was

TABLE II

RELATIVE MOLAR RATIO OF THE PARTIALLY METHYLATED MANNOSE DERIVED FROM THE ENZYMIC REACTION WITH 1,2-α-D-MANNOSIDASE FROM *ASPERGILLUS SAITOI* AT PH 5.0

east mannan	Enzymic reaction time (min)	Relative molar ratio		
		Methyl-2,3-4,6-tetra-*O*-methyl-D-mannoside (Relative retention time, 1.00)	Methyl-3,4,6-tri-*O*-methyl-D-mannoside (Relative retention time, 1.99)	Methyl-2,4,6-tri-*O*-methyl-D-mannoside (Relative retention time, 2.42)
ker's yeast	0	1.00	0.46 (1.00)	0.43 (1.00)
	30	1.00	0.36 (0.79)	0.42 (0.98)
	120	1.00	0.35 (0.76)	0.40 (0.94)
ké yeast	0	1.00	0.42 (1.00)	0.78 (1.00)
	30	1.00	0.37 (0.89)	0.73 (0.94)
	120	1.00	0.35 (0.84)	0.76 (0.97)

recognized that the molar ratio of 1,2-α- and 1,3-α-linked side chains of baker's yeast mannan was 1 : 1 [19,20]. Kumagai et al. [10] found that the saké yeast mannan had a similar structure to that of baker's yeast mannan. However, some differences were found in their branch structures. In the saké yeast mannan, twice as much 1,3-α-linkage was found as 1,2-α-linkage.

According to the experimental results of Bhattacharjee and Gorin [18], methyl-2,3,4-tri-*O*-methyl-D-mannoside could be separated from methyl-3,4,6-tri-*O*-methyl-D-mannoside. In Fig. 6 of the present study, we could not detect methyl-2,3,4-tri-*O*-methyl-D-mannoside. From the studies of the structure of baker's yeast mannan, our results in Fig. 6 can be explained in that the baker's yeast mannan is a highly branched polysaccharide with 1,6-α-linked backbone, and that there are few 1,6-α-linked backbone mannoses in the baker's yeast mannan without 1,2-α- and 1,3-α-linked side chains.

It was assumed that the enzyme could not cleave 1,3-α-linkages and 1,6-α-linkages in these substrates, because decrease of methyl-2,4,6-tri-*O*-methyl-D-mannoside and methyl-2,3,4,6-tetra-*O*-methyl-D-mannoside was not observed on prolonged incubation.

The time course of decrease of methyl-3,4,6-tri-*O*-methyl-D-mannoside in Table II shows that the reaction for decrease of methyl-3,4,6-tri-*O*-methyl-D-mannoside was initiated with a rapid hydrolysis. It was assumed from the Fig. 6 and Table II that the resistant polymer from the enzymic product could be a 1,6-α-linked core-polymer containing mainly 1,3-α-linked side chains and 1,2-α-linked di- and trisaccharides with terminal 1,3-α-D-linked side chains. The results obtained by the present studies with yeast mannan suggested that *A. saitoi* α-D-mannosidase hydrolyzes only Man α1 → 2 Man linkage.

The K_m value for the baker's yeast mannan from a Lineweaver-Burk plot was 0.45 mM, based on a molecular weight of 22 000 [5].

Discussion

A. saitoi α-mannosidase described in this report is active on yeast mannan, producing free mannose. Many α-mannosidases are known and, of these, the enzymes of rat epididymis [21], jack bean meal [22], marine gastropods [23], *S. cerevisiae* [24] and *A. niger* [9] have been studied extensively. Significant differences exists between these enzymes and *A. saitoi* α-mannosidase described in this paper. One notable difference is that *A. saitoi* α-mannosidase was not active on α-mannoside with aromatic aglycon, *p*-nitrophenyl-α-mannoside, whereas the compound is a good substrate for the other five enzymes. This finding could be related to the different metabolic roles of the various enzymes.

A. saitoi α-mannosidase, on the other hand, exists presumably for the purpose of digesting yeast mannan. The glycosidic linkages in this substrate are adjacent almost exclusively to other polar mannose residues. Thus, the α-mannosidase from *A. saitoi* might be expected to be less active on mannoside containing nonpolar aglycon.

Another significant difference between *A. saitoi* α-mannosidase and the 1,2-α-mannosidase from *A. niger* reported by Matta and Bahl [9] is that *A. saitoi* α-mannosidase was active on baker's yeast mannan, whereas the yeast

mannan is not a substrate for the 1,2-α-mannosidase from *A. niger*.

Studies with the oligosaccharides obtained by acetolysis of *S. cerevisiae* have shown that *Arthrobacter*-α-mannosidase [6] cleaves 1,2-α-, 1,3-α- and 1,6-α-linkages in these substrates, releasing a mannose residue from their non-reducing ends [6]. *Arthrobacter* α-mannosidase was not able to hydrolyze myoinositol-2-*O*-α-D-mannoside. This finding suggests that the *Arthrobacter* α-mannosidase is very sensitive to the structure of the aglycon.

Preliminary results have confirmed, by incubation with radioactive oligosaccharides, that *A. saitoi* α-mannosidase preparation is free from contamination with β-mannosidase, β-galactosidase, β-*N*-acetylhexosaminidase and α-L-fucosidase [25]. None of the substrate oligosaccharides was hydrolyzed. The results obtained by the present studies with baker's yeast mannan suggested that *A. saitoi* α-mannosidase hydrolyzes only Man α1 → 2 Man linkage. For further confirmation of this specificity, the action of *A. saitoi* α-mannosidase on a series of small oligosaccharides, Man α1 → 2 Man α1 → 3 Man β1 → 4 GlcNAc, Man α1 → 3 Man β1 → 4 GlcNAc and Man α1 → 6 Man β1 → 4 XylNAc was investigated [25]. The preliminary results showed that *A. saitoi* α-mannosidase can hydrolyze only Man α1 → 2 Man α1 → 3 Man β1 → 4 GlcNAc at the Man α1 → 2 Man linkage [25]. The K_m and V values were calculated as 0.8 mM and 10 μmol/min per mg enzyme, respectively.

It can be concluded that *A. saitoi* α-mannosidase is highly specific for 1,2-α-D-mannosidic linkage and therefore readily hydrolyzes baker's yeast mannan. The trivial name *A. saitoi* 1,2-α-D-mannosidase is suggested for this new α-mannosidase.

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